Characterization of the biomechanics of the GPIbα-vWF tether bond using von Willebrand Disease causing mutations R687E and wt vWF A1A2A3

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Platelet aggregation plays an important role in controlling bleeding by forming a hemostatic plug in response to vascular injuries. GPIbα is the platelet receptor that mediates the initial response to vascular injuries by tethering to the von Willebrand factor (vWF) on exposed subendothelium. When this occurs, platelets roll and then firmly adhere to the surface through the GPIIb-IIIa integrin present on the platelet surface. A hemostatic plug then forms by the aggregation of bound and free platelets which then seals the injury site.

vWF is a multimer of many monomers, with each containing eleven domains. In this experiment, biomechanics of two of the eleven domains, gain of function (GOF) R687E vWF-A1 and wild type (wt) vWF-A1A2A3, were studied using videomicroscopy under varying shear stresses. This experiment used a parallel flow chamber coated along one surface with the vWF ligand. A solution containing platelets or Chinese Hamster Ovary (CHO) cells was perfused at varying shear stresses (0.5 dynes/cm2 to 512 dynes/cm2) and cell-ligand interactions were recorded.

Results showed that GOF R687E vWF exhibited slip bond behavior with increasing shear stress, whereas wt A1A2A3 vWF displayed a catch-slip bond transition with varying shear stresses. Interestingly, wt A1A2A3 vWF displayed two complete cycles of catch-slip bond behavior, which could be attributed to the structural complexity of the vWF ligand. However, more experiments need to be performed to further substantiate these claims. Information on the bonding behavior of each vWF can aid understanding of the biomechanics of the entire vWF molecule and associated diseases.

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INTRODUCTION

Circulating platelets have an important role in healing vascular injuries by tethering, rolling, and adhering to the vascular surface in response to a vascular injury. Under normal physiological conditions, platelets respond to a series of signaling events that cause bound platelets to aggregate and spread across the exposed surface to form a hemostatic plug (Andrews, 1997). These responses are mediated by receptor-ligand interactions between the platelet and the molecules exposed on the surface. GPIbα is the platelet receptor that mediates this initial response to vascular injuries. In arteries this response is initiated when platelet receptor GPIbα tethers to von Willebrand factor, a blood glycoprotein, on exposed subendothelium—the surface between endothelium and artery membrane. When GPIbα initially tethers to von Willebrand factor (vWF), platelets first roll and then firmly adhere to the surface through the GPIbα and GPIIb-IIIa integrins present on the platelet. GPIbα and GPIIb-IIIa integrins are the first two platelet integrins to interact with vWF molecule (Kroll, 1996). Aggregation of bound platelets with additional platelets from the plasma forms a hemostatic plug that seals the injury site (Ruggeri, 1997).

Mutations in either of these binding partners can result in changes in the initial step of the vascular healing process. Diseases associated with these mutations are called von Willebrand diseases, which can either decrease (loss of function) or enhance (gain of function) the binding activity between the GPIbα and vWF molecules. von Willebrand diseases (VWD) result in a platelet dysfunction that can cause nose bleeding, skin bruises and hematomas, prolonged bleeding from trivial wounds, oral cavity bleeding, and excessive menstrual bleeding. Though rare, severe deficiencies in vWF can have symptoms characteristic of hemophilia, such as bleeding into joints or soft tissues including muscle and brain (Sadler, 1998).

vWF is a multimer of many monomers, with each containing eleven (11) domains (Figure 1) (Berndt, 2000). In this experiment, biomechanics of two of the 11 domains, in particular, gain of function (GOF) R687E vWF-A1 and wild type (wt) vWF-A1A2A3 were studied. Biomechanics of the GPIbα-vWF tether bond of these molecules was studied using videomicroscopy in parallel plate flow chamber experiments. One of the two surfaces of the flow chamber was a 35-mm tissue culture dish coated with the vWF ligand (Figure 2). Fluid con-

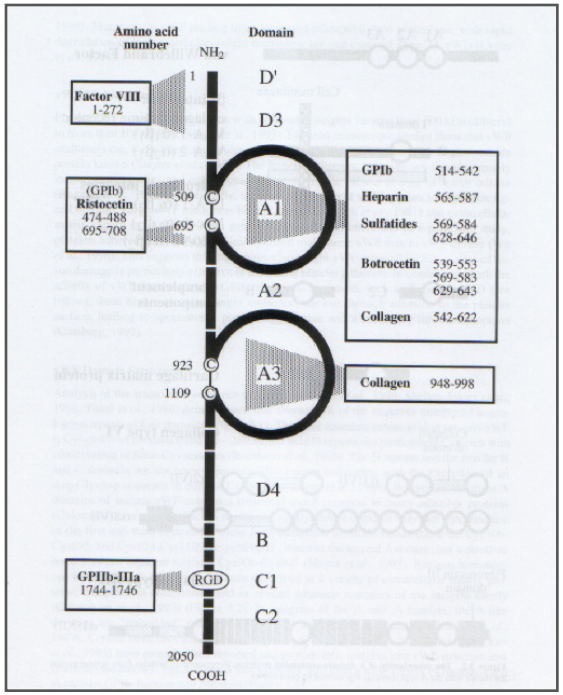

Figure 1. The vWF molcule. It is a multimer of many monomers, with each containing 11 domains. Image adapted from Sadler.

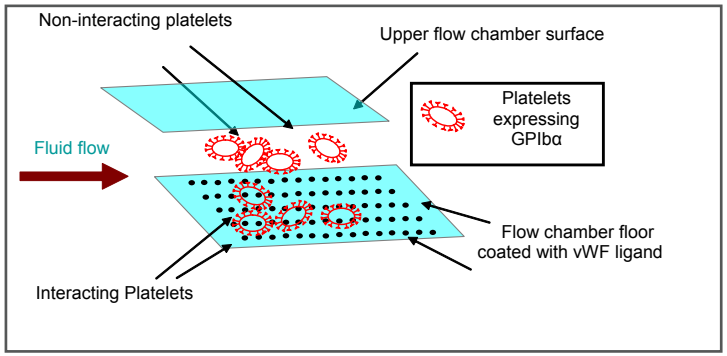

Figure 2. TParallel plate flow chamber setup. The floor (bottom) plate in the setup was a 35-mm tissue culture dish coated with vWF ligand. Fluid containing either CHO cells or platelets was perfused at varying shear stresses (0.5 dynes/cm^2 to 512 dynes cm^2) across the ligand coated surface.

taining either platelets or Chinese Hamster Ovary cells were perfused at varying shear stresses across this ligand coated surface and the interactions were recorded using high speed videomicroscopy. Analysis of these interactions with cell tracking software allowed insight on bond lifetime of the cells and helped suggest the type of bond present (Yago, 2004).

By studying the biomechanics of individual vWF domains, it will allow a better understanding of the whole vWF molecule, and more importantly, VWD. With this enhanced understanding of vWF, better and more accurate treatments for VWD can be designed in future. This knowledge can also be used in studying and preventing life threatening thrombosis and embolism.

MATERIALS AND METHODS

All materials were obtained from the McIntire laboratory stock room. Proper sterile techniques and precautions were used for each of the following procedures.

Cells Used

Either Chinese Hamster Ovary (CHO) cells or fresh platelets were used to study the vWF ligand interactions. Fresh platelets were isolated from blood donors an hour before the experiment. For CHO cells, two specific lineages, αβ9 and β9, were used. CHO αβ9 cells contain specific integrin that interact with the vWF ligand, whereas β9 cells do not. Hence, β9 cells served as a control group when CHO cells were used instead of platelets.

Preparation of groth media

Two types of growth media were prepared for the two types of CHO cells: αβ9 and β9. Both media formulations consisted of alpha-Minimum Essential Medium (α-MEM) solution (with 2mM L-glutamine and NaHCO3), 10% Fetal Bovine Serum (FBS) solution, penicillin solution (50X), streptomycin solution (50X), G-418 (Geneticin) solution (50 mg/mL), and methotrexate powder. The only difference between the two media types was the addition of hygromycin B solution (50 mg/mL) in the αβ9 media.

Passaging cells

Proper sterile techniques and precautions were used while passaging CHO αβ9 and CHO β9 cells. CHO cells were cultured in 75 cm^2 flasks and incubated at 37° Celsius, 5% CO2 using the growth media prepared. These cells were passaged every 2-3 days in order to maintain 80-90% confluency of cells at all time.

Hepes-Tyrode buffer formations

Hepes-Tyrode buffer (also referred as 0% Ficoll) was pre-

pared by mixing the following chemicals in pure deionized water until completely solvated: Sodium chloride (135 mM), sodium bicarbonate (12 mM), potassium chloride (2.9 mM), sodium phosphate monobasic (0.34 mM), hepes (5 mM), glucose D (5 mM), and BSA (1% weight per volume). CHO cells and platelets were suspended in this buffer for the flow chamber experiments. This solution consisting of cells and buffer was pumped through the flow chamber at various shear stresses.

Ficoll Solution

A more viscous Hepes-Tyrode buffer was prepared by adding 6% Ficoll (weight per volume). The final viscosity was 1.8 times that of Hepes Tyrode buffer. This ficoll solution is also referred as 6% Ficoll.

Parallel plate flow chamber experiments

A parallel plate flow chamber was used in this experiment. One of the two surfaces of the flow chamber was a 35-mm tissue culture dish coated with the vWF ligand. Fluid containing either CHO cells or platelets was perfused at varying shear stresses (0.5 dynes/cm^2 to 512 dynes/cm^2) across the ligand coated surface (Figure 2). The interactions were recorded as 4-second videos at 250 frames/second using high speed videomicroscopy (Figure 3). The parallel plate flow chamber set-up was maintained at 37° Celsius for all experiments.

Tracking cell interactions

MetaMorph Offline software was used to track the interactions captured with videomicroscopy. Each 4-second video was opened using this software and a square was drawn around the CHO cell or platelet (hereon referred as "cell") of interest. Each cell was tracked for at least 250 continuous frames (1 second). In addition, it was ensured that no other cell bumped into the cell of interest while it was being tracked by observing the video.

Data analysis

The tracked results from MetaMorph Offline were saved as multiple number strings in Microsoft Excel and processed through MATLAB to compute mean rolling velocities for each shear stress. The mean rolling velocity suggests how fast the cell is rolling while interacting with the vWF ligand at each individual shear stress. These rolling velocities were graphed in Microsoft Excel for each shear stress in a logarithmic scale.

RESULTS

GPIbα and von Willebrand factor (vWF) interactions were recorded using videomicroscopy in parallel plate flow chamber experiments. These interactions were then tracked using MetaMorph Offline, the tracking software, for at least 250 continuous frames (1 second).

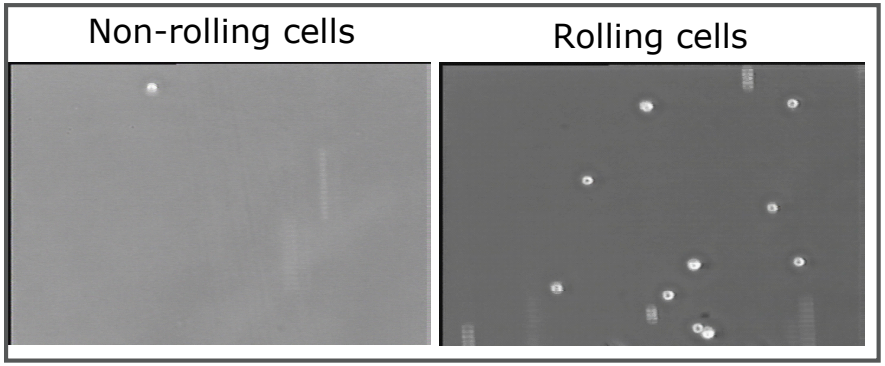

Figure 3. A snap shot of the videomicroscopy recording of CHO cells interacting on a vWF coated surface. The non-rolling cells free flow over the surface without any interactions. In contrast, the rolling cells are visibly flowing slower as they interact with the vWF ligand.

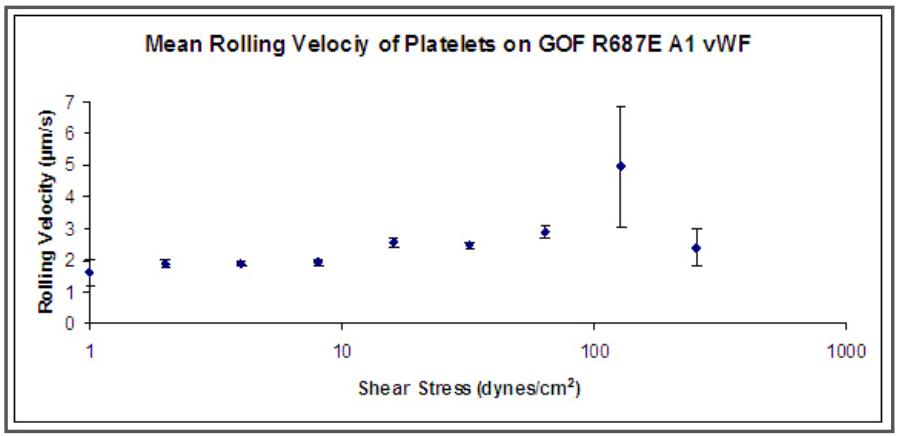

Figure 4. A mean rolling velocity of platelets on gain on function (GOF) R687E A1. The x-axis represents the logarithmic shear stress (dynes/cm^2) while the y-axis represents the mean rolling velocity (µm/s). The error bars represent the Standard error of means. SEM. Increasing velocity suggests a slip bond behaviors of GOF R687E.

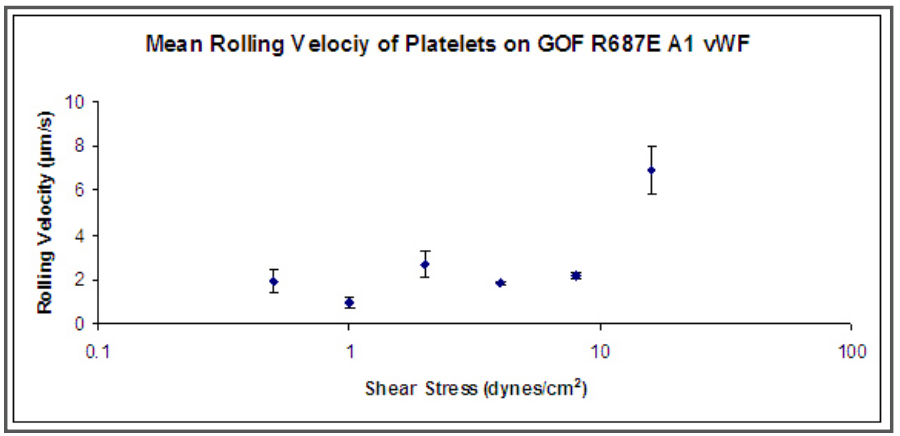

Figure 5. Mean rolling velocity of platlets on gain of function (GOF) R687E A1. The x-axis represents the logarithmic shear stress (dynes/cm^2) while the y-axis represents the mean rolling velocity (µm/s). Error bars represent the Standard error of means, SEM. The increasity velocity suggests a slip bond behavior of GOF R687E.

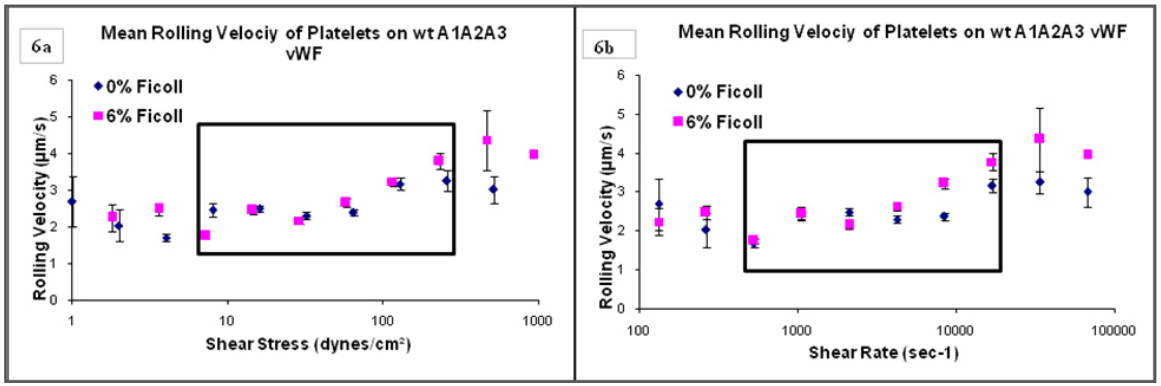

Figure 6a and 6b. Mean rolling velocity platelets on wt A1A2A3 vWF. Y-axis in both 6a and 6b represent the mean rolling velocity (µm/s), whereas the x-axis in 6a represents the logarithmic shear stress (dynes/cm^2) and in 6b represents the logarithmic shear rate (s-1). The error bars represent the Standard error of means, SEM. This cycle of decreasing and increasing mean rolling velocity is indicative of a catch-slip bond interaction between GPIba and vWF ligand.

The results from MetaMorph Offline were processed through MATLAB to compute the mean rolling velocities for each shear stress used (0.5 dynes/cm^2 to 512 dynes/cm^2). In order to learn about the GPIbα-vWF tether bond interaction, mean rolling velocities were plotted versus shear stress for each individual experiment.

Plotting the results for platelets interacting on gain of function (GOF) mutant R687E vWF-A1 molecule revealed a trend of increasing mean rolling velocities with increasing shear stress (Figure 4). The x-axis represents the logarithmic shear stress (dynes/cm^2) while the y-axis represents the mean rolling velocity (µm/s). The error bars are the standard error of mean (SEM), which is calculated by dividing the standard deviation by the square root of number of samples (stdev/$\sqrt{(N)}$).

Intuitively, with increasing shear stress the bond lifetime decreases for each individual bond (one-to-one molecule interaction between GPIbα and vWF ligand); consequently, causing the mean rolling velocity to increase at higher shear stresses. This increase in mean rolling velocity is characteristic of a slip bond interaction, because the molecules tend to "slip off" the ligand more readily at higher shear stress than at lower shear stress.

A separate experiment performed with platelets from a different donor on GOF R687E vWF showed a similar slip bond interaction (Figure 5). Although less data points were collected in this experiment, it showed a similar increase in mean rolling velocity with increasing shear stress. A statistical analysis of these two data sets revealed a pearson correlation factor of 0.98 and a p-value greater than 0.05 for a paired t-test. Therefore, reproducibility of this trend affirmed the slip bond characteristic of GOF R687E vWF-A1 molecule.

Outliers at high shear stress are attributed to the fact that bond lifetime significantly decreases at higher shear stress. As a result, fewer platelets interact at those shears and thus fewer data points were collected at higher shear stress compared to at lower shear stresses. This is reflected with the large SEM bars for data points at higher shear stress end. Similarly, mean rolling velocity at the lowest shear stress are also variable because of

the diffuclty in distinguishing interacting platelets from non-interacting platelets. For both experiments, platelets were suspended in Hepes Tyrode buffer.

In contrast, platelets interacting on wild type (wt) A1A2A3 vWF molecule (Figures 6a and 6b) showed a different trend compared to GOF R687E vWF. Y-axis in both Figures 6a and 6b represent the mean rolling velocity (μm/s), whereas the x-axis in 6a represents the logarithmic shear stress (dynes/cm^2) and in 6b represents the logarithmic shear rate (s-1). The error bars represent the SEM. As illustrated by the graphs, mean rolling velocity initially decreased, then increased and decreased only to increase again with increasing shear stress (and shear rate). This cycle of decreasing and increasing mean rolling velocity is indicative of a catch-slip bond interaction between GPIbα and vWF ligand.

A decrease in mean rolling velocity correlates with an increase in bond lifetime of individual bond, and thus indicating a catch bond because the platelet is "caught" by the ligand. Likewise, an increasing mean rolling velocity implies a decrease in bond lifetime of the individual bond as a slip bond interaction. Platelets on wt A1A2A3 vWF exhibited two complete cycles of catch-slip bond interaction for the range of shear stress measured (0.5 dynes/cm^2 to 512 dynes/cm^2). For this particular experiment, platelets were suspended in Hepes Tyrode buffer (0% Ficoll) and 6% Ficoll solution. Suspending platelets in a more viscous solution was used to verify whether the catch-slip bond was force dependent or transport dependent.

A similar catch-slip bond interaction was illustrated with Chinese Hamster Ovary (CHO) cells interacting on wt A1A2A3 vWF (Figure 7). Although fewer data points were collected for this experiment, it still demonstrated two cycles of decreasing and then increasing mean rolling velocity with increasing shear stress. No statistical analysis was performed between these two results because CHO cells contain isolated GPIbα receptors, whereas platelets have many molecules on their surface. Thus, the mean rolling velocities are comparably different between them and not comparable.

DISCUSSION

Fresh platelets and wild type (wt) Chinese Hamster Ovary (CHO) cells were used on gain of function (GOF) R687E vWF or wt A1A2A3 vWF in order to study some aspects of the GPIbα-vWF tether bond. Parallel plate flow chamber experiments were the same for each vWF molecule. The only difference was the fluid passing through had either platelets or wt CHO cells. All rolling interactions were observed at 250 frames per second.

It was previously found that wild type-wild type (wt GPIbα on wt vWF) interactions differ from wt-GOF (wt GPIbα on GOF vWF) interactions. An additional experiment (Appendix A, Figure A1) shows platelets on wt vWF-A1. This graph shows a transition of bonding behavior from a region of decreasing rolling velocity to an increasing rolling velocity as the shear stress increases. This trend is indicative of a catch-slip bond transition because the rolling velocity decreases (catch behavior) and then increases (slip behavior) with increased shear stress.

However, results from platelets on GOF R687E vWF (Figures 4 and 5) showed an increase in rolling velocities with increased shear stress—indicating only a slip bond behavior. This suggests that a catch bond governs low force binding behavior between wt GPIbα and wt vWF-A1; whereas a slip bond governs binding of GOF R687E at high shear stresses. One possible reason for this could be the differential force response of the bond lifetime.

Results of platelets rolling on wt A1A2A3 vWF (Figures 6a and 6b) showed two complete cycles of bonding

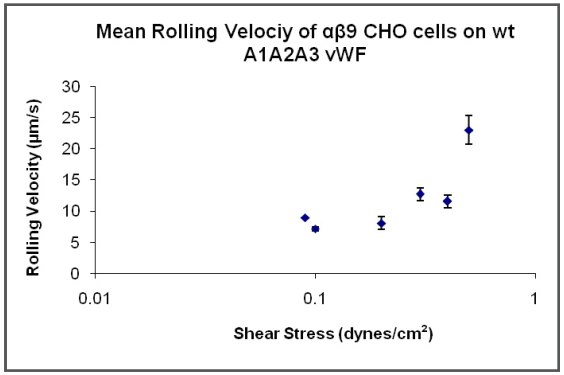

Figure 7. Mean rolling velocity of CHO cells on wt A1A2A3 vWF. X-axis represents the logarithmic shear stress (dynes/cm^2) while the y-axis represents the mean rolling velocity (µm/s). The error bars represent the Standard error of menas, SEM. This cycle of decreasing and increasing mean rolling velocity is indicative of a catch-slip bond interaction between GPlba and vWF ligand.

behavior from a region of decreasing rolling velocity to increasing rolling velocity as shear stress increased. This cycle of decreasing and increasing mean rolling velocity is indicative of a catch-slip bond interaction between GPIbα and vWF ligand. When rolling velocity decreases, it is indicative of a catch bond, suggesting the bonds are stuck or caught on the ligand and hence slowing its velocity. Likewise, when the rolling velocity increases, it indicates a slip bond because the bond comes off or slips off much quicker and consequently increases the rolling velocity. Based on previous knowledge (Figure A1), this catch-slip bond behavior can be identified with the presence of wt A1 thedomain in the wt A1A2A3 vWF ligand. However, two observed complete cycles of catch-slip bond might be due to the structural complexity of the complete A1A2A3 vWF ligand.

Also, viscosity of the fluid in which platelets were suspended was increased by 1.8 times. By comparing the results from these two different solutions (0% Ficoll and 6% Ficoll), it helped determine whether if this catch-slip bond interaction was force dependent or transport dependent. Figures 6a and 6b show a boxed

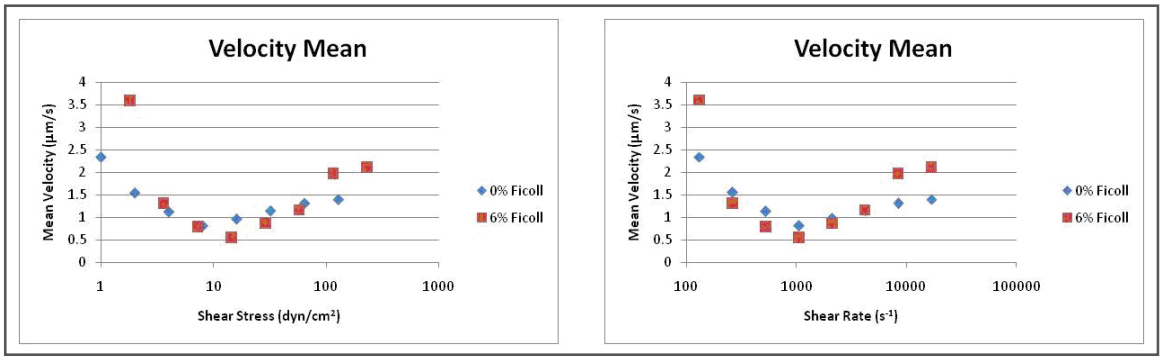

Figure A1. Results of platelets on wt-A1 vWF. Y-axis in both left and right plots represent the mean rolling velocity (µm/s), whereas the x-axisin left represents the logarithmic shear stress (dynes/cm^2) and in right represents the logarithmic shear rate (s-1). These plots show a catch-slip bond interaction, the the rolling velocity decreases and then increases with increases shear stress (and shear rate).

region where the data points for the two different solutions (with 0% Ficoll and 6% Ficoll) align or overlap when plotted together. Since shear stress data aligns the best compared to shear rate data, it indicates that force, which regulates shear stress, is probably what governs this catch-slip bond interaction.

A similar catch-slip bond interaction was observed between wt CHO cells on wt A1A2A3 vWF (Figure 7). The bond behavior transitions from a region of decreasing rolling velocity to a region of increasing rolling velocity. CHO cells have isolated GPIbα receptors, which allows for the isolation of the GPIbα receptor's contribution to the rolling velocity parameter, since platelets have many molecules on their surface. Thus, this trend could be attributed to the GPIbα receptor's interactions with the vWF molecules and A1A2A3 structure.

Overall, bond behaviors of the two vWF domain, GOF R687E and wt A1A2A3, were successfully characterized. Although the bonding trends of the vWF ligand appear very obvious, more testing will help further substantiate these claims. Based on the results, the next step would be assessing how these bond types adversely affect platelet aggregation in presence of a vascular injury. By determining the adverse effects of different bond type in each vWF domain, it will further help understand VWD and its causes and potentially lead to a treatment.

CONCLUSION

Some valuable information on tether bonding between GPIbα-vWF, specifically GOF R687E vWF and wt A1A2A3 vWF, was acquired from the four set of experiments performed. Results from two experiments revealed a pure slip bond behavior for platelets rolling on GOF R687E (Figure 4-5). Statistical analysis also showed a strong correlation and p-value greater than 0.05 between the two experiments involving GOF R687E vWF; hence, confirming the reproducibility of slip bond behavior. This slip bond behavior is attributed to the differential force response of bond lifetime between GPIbα and GOF vWF ligand with increasing shear stress.

In addition, studying wt A1A2A3 vWF on platelets and wt CHO cells revealed two complete cycles of catch-slip bond behavior (Figure 6-7). Based on previous knowledge, this catch-slip bond behavior can be identified with the presence of wt A1domain in A1A2A3 ligand. However, having two cycles of catch-slip bond behavior can be due to the structural complexity of A1A2A3 vWF ligand.

In future studies, more experiments need to be performed with wt A1A2A3 vWF on platelets and CHO cells in order to confirm the reproducibility of the results achieved. More data is needed to support the claim that having two cycles of catch-slip bond can be attributed to the structural complexity of A1A2A3 vWF ligand. Similarly, more experiments involving GOF R687E vWF on platelets and CHO cells will further substantiate the slip bond behavior of GOF vWF. By studying biomechanics and bond behavior of each domain of the vWF molecule, it will allow a better understanding of vWF and VWD.

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